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Characterization of the volatile odor profile from larval masses in a field decomposition setting

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ABSTRACT

By studying insect populations and subsequent larval stages, forensic examiners can use entomology as a tool to estimate time of death, evaluate trauma present in body, and even determine if the corpse was moved based on the types of insects found. Due to the close contact between insect and decomposition source, this study aimed to investigate whether a larval mass sample could yield an odor profile distinctive of the various stages of decomposition using pig cadavers as models.

Instrumental evaluation utilized Solid Phase-Microextraction (SPME) coupled with Gas Chromatography-Mass Spectrometry (GC–MS) for the identification of extracted volatile odor profiles of maggots. There were 107 compounds detected in larvae samples. Of these, a total of 10 compounds were selected as frequently occurring in the larvae matrix. Sulfurs and ketones were observed in the early stages of decomposition, followed by alcohols such as phenol and indole in later stages. Based on the analysis of released volatile organic compounds, it is feasible to use a larval odor sample to detect previously reported decomposition odor volatiles and through continuous sampling, the odor profile changes as a function of decomposition. Principal component analysis depicted a preliminary decomposition stage clustering of larvae odor profiles using only the selected volatile array pattern. More research is therefore needed to confirm this trend over longer longitudinal and temperature perspectives. This study, however, provides an initial foundation as to how these larval mass odor profiles could provide a preliminary path for a new tool in PMI determination.

1. Introduction

Forensic entomology uses insects and other arthropods found on decomposing cadavers to include specialized areas of study such as calculation of insect life-stage development timeframes, faunal succession, collection and analysis of insect evidence to identify the victim and possible movement of the corpse, as well as the main use of forensic entomological procedures – aid in the determination of time since death, commonly referred to as the postmortem interval (PMI) [1]. For early PMI stages, algor, livor, and rigor mortis along with gastric contents are useful and commonly used [2]. For late PMI, insect development proves to be the technique of choice for estimating the minimum PMI [3]. Various methods are globally implemented to calculate postmortem interval using forensic entomological evidence. Succession of insects on the corpse determine time patterns, as different species arrive on the corpse depending on the body's decomposition stage This is referred to as insect succession waves. By just looking at the succession waves of insects, it can be determined what stage of decomposition a corpse is in. Furthermore, developmental patterns and weights of larvae are used with statistical models to determine larval age. Recently, DNA techniques have been exploited to obtain genetic fingerprints for specimen, age-dependent differential gene expression and species identification as well as to isolate human DNA from insect gastrointestinal contents [4–6]. The crime scene where the body is recovered plays a pivotal role in the types of insects found as well as offers external factors that influence entomological evidence such as: temperature, composition of soil, humidity, clothing, and other intrinsic properties [7–14].

One novel area of study in forensic entomology has involved identifying volatile organic compounds released by larvae and pupae of insects. The focus has been on the profiling of the cuticular hydrocarbon composition as a function of time from puparia samples of different ages under laboratory conditions [15–19]. Recently, studies have extended this research area by increasing the targeted compounds analyzed to include fatty acids and hydrocarbon composition [20] as well as the

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overarching volatile profile patterns across various functional groups [21]. These studies have demonstrated that there are differences in the volatile composition which can assist in species identification and can be used to provide an additional taxonomic tool.

Other studies have targeted volatile compounds with respect to entomological samples as a function of cadaver preferences. For this purpose, buried cadavers have been used to attract insects to see whether above or below ground cadavers have a faster decomposition rate [22]. Researchers have also studied insect succession on carrion with pig cadavers as a model to help determine the insect sequence over a period of time [23]. There has also been research that tested what chemical compounds where involved in the discrimination and recognition of specific stages of decomposition using gas chromatography coupled to electroantennography to identify the target compounds within a beetle species that aid in cadaver location for feeding. [24]. Others have observed the electrophysiological and behavioral responses in male and female *Thanatophilus sinuatus* [25]. In this article, there were a few cadaveric volatile organic compounds tested including: dimethyl disulfide (DMDS), butan-1-ol, n-butanoic acid, indole, phenol, p-cresol, putrescine, and cadaverine [25]. Of these cadaveric compounds tested, butan-1-ol and dimethyl disulfide (DMDS) emitted the strongest electroantennography (EAG) from both *T. sinuatus* male and female antennae [25].

One current research gap within the use of volatile organic compounds and larva samples is that all studies have focused on the headspace analysis of entomological samples that have been reared in the laboratory with meat samples as the substrate for oviposition. No current study to date has sampled larval samples directly from a decomposing cadaver for volatile profiling. Hence, the purpose of this study was to establish an animal model for blow fly larval headspace sampling purposes to determine specific chemical compounds as a function of the decomposition stage of the piglet cadaver. The aim of the study is to investigate qualitative and semi-quantitative differences in the detected odor profile above the headspace of a maggot collected off a cadaver at different decomposition time intervals. As previous studies focused on specific hydrocarbons, this study geared to collect maggots from infested piglet cadavers under field conditions over the entire decomposition period to determine the volatile odor patterns across time with no specific functional group target, but rather obtain a general volatile odor profile identification from the blow fly (Diptera: Calliphoridae) larvae thereby providing an additional tool to enhance PMI estimations.

2. Methods and materials

2.1. Piglet carcasses

Eight pig carcasses were utilized as human analogues in this study (0.5 kg–1.5 kg). Experimental logistics dictated the size of the carcasses available for this study. It can be noted that even though the weight is not representative of a human cadaver, they did provide an active decomposition source in an outdoor setting. Pigs were chosen as the target model due to their physiological similarities to humans [26]. No ethical permissions were required as the pigs were not euthanized for the purpose of this study, but were kindly donated by hunters in the area. Appropriate notification was given to the Institutional Animal Care and Use Committee (IACUC) which determined a full protocol was not required and issued a non-protocol #X18093 for this study. Two pigs were used during the pilot study for method development, and six pigs for the experimental runs of the study (Table 1). Due to logistical reasons, the pigs were stored in a laboratory freezer at −26 °C before use due to carcass donations before experimental set up. Before laying out the pigs on the field test site, they were completely defrosted. Although it is acknowledged that this procedure can cause an alteration to the animal model [27], literature has highlighted that reduced bacterial activity at lower temperatures simulates limited bacterial activity of neonatal remains [28]. Table 1 summarizes the dates of carcass placement for decomposition processing.

All carcasses were left to decompose in an outdoor private field site in Levelland, TX. The pigs were laid out on the ground approximately five feet apart to make sure no interferences between pigs and their decomposition processes occurred. A homemade cage covered the pigs to secure from larger scavengers (i.e., raccoons and coyotes). Each pig had its own cage to provide protection.

The insect that was targeted for this study is the maggot of a Blow Fly (Calliphoridae) [29]. A genus species was named once there was knowledge of what flies were prominent on the cadavers. Sampling occurred for a period of 5 to 26 days, where collection was completed once a day, every 24 h from the time the pig was laid out. These time intervals were edited during the pilot study due to the optimal time maggots were observed.

2.2. Pilot study

There were two pigs used for the pilot study. One pig was laid out at a time to document the decomposition process and the length of time it would take for full decomposition. Maggot samples were collected throughout this process to test the feasibility of obtaining VOCs from the headspace of such samples and to monitor the types and abundances of VOCs as a function of decomposition stage. Only four of the five of the stages of decomposition were targeted for sampling. These stages included: fresh, bloat, and active decay, and advanced decay as these are the stages where maggots are still feeding on corpse and have not migrated away from carcass to pupate [30]. Three regions of the pig were also targeted throughout the pilot study to evaluate which region had the most abundant maggot population. The regions were divided into 3 main parts: top cadaveric region, central cadaveric region, and rear cadaveric region, as seen in Fig. 1. Once the first pig had decomposed completely, it was removed, and a second pig was laid in its place. In the pilot study, the same location was used for the pigs. However, during the test run, distinctive locations were used for each pig carcass. The process was completed for both carcasses. A total of ten samples were collected during each collection time if available for each pilot pig (three top, three central, three rear, and one tissue sample).

The temperature, humidity, soil pH, and soil moisture were measured and recorded during each sampling time during both pilot and experimental runs. Temperature fluctuations during all the experimental study can be seen in Fig. 2. A digital thermometer and stainless-steel probe were used to monitor daily temperature and humidity conditions at field site (Fisher Scientific), both within the soil and above soil conditions. Insects were collected in capped vials for easy transfer to the laboratory. The instrumental analysis of the collected samples was done within three days of collection. All sample specimens were kept at room temperature during extraction and analysis procedures.

The control for this study was taking concurrent samples from the pig carcass (i.e. skin) to compare VOCs given off from other traditional sample sources during the pig's decomposition. Only one skin sample was collected from the pig cadaver as a control. However, the control sample matrix did change as a function of decomposition stage as the skin began to become not available. This information was then compared to the VOCs analyzed from the maggot samples.

All samples collected (i.e., maggot and pig tissue samples) were weighed. The weights were documented and used to standardize the samples to 1 g per maggot/pig tissue. This was done by dividing the peak abundance of each compound by the weight of the maggot/tissue sample collected during that sampling time. This ensured that each sample was standardized throughout the study for a uniform comparison of peak abundances for each compound detected. Standardizing the weights of each sample equalized the data variability.

2.3. Experimental runs

Larval and tissue samples were only collected from the central

Table 2
Medium and High Frequency Volatile Compounds Released by Larvae & Tissue Samples Over Time.

		Week 1		Week 2						Week 3				Week 4		Frequency	Previously Reported Literature
Compound	Retention Time (~)	M1	T1	M1	T1	M2	T2	M3	T3	M2	T2	M3	T3	M3	T3	#	
Sulfur																	
Dimethyl disulfide	4.563	X	X	X	X	X	X	X		X	X	X	X	X	X	13	[8,25,31,36,40,41,43,44,45,48,50,51,52,53,54,55,56,57,59,61,62,65,66]
Dimethyl trisulfide	8.925	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14	[8,36,38,39,41,44,45,47,48,50,52,55,56,57,59,60,61,63,64,65]
Dimethyl tetrasulfide	11.382					X	X			X	X	X	X			6	[39,48,52,55,65]
Alcohol																	
Phenol	9.093	X	X	X	X	X	X	X		X	X	X	X	X	X	13	[8,21,25,33,37,39,41,47,48,49,50,52,55,59,64]
Hexanol	9.548	X	X	X	X	X		X						X	X	8	[8,29,39,52,55]
Phenylethyl alcohol	10.415	X	X	X	X	X	X		X	X	X	X	X	X	X	13	[39]
Benzyl alcohol	9.644	X	X			X	X		X		X		X			7	[33,39,47]
Nonanol	10.847	X	X				X		X	X	X		X	X		8	[58]
Butanol	4.298	X	X				X		X		X		X			6	[38,39,43,44,48,52,55,65]
p-Cresol	10.131	X	X	X		X	X	X	X	X	X	X	X	X	X	13	[64]
Propanol	9.11		X				X		X	X			X			5	[8,38,44,45,46,55,56,57,65]
Octanol	9.944	X	X						X		X		X		X	6	[39,47,52,65]
Ketone																	
Octanone	9.104		X		X		X		X				X		X	6	[38,48,52,65]
Acetophenone	11.974	X	X		X	X	X	X		X	X	X	X	X	X	12	[41,50,55]
Nonanone	10.159	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14	[8,31,52]
Undecanone	11.811	X	X	X	X	X	X	X	X	X		X	X	X	X	13	[31,32,52]
Ethanone	11.971	X	X	X	X	X										5	[8,47,65]
Tridecanone	13.149	X		X	X	X	X	X		X		X			X	9	
Undec-6-en-2-one	11.697	X			X	X		X				X	X	X	X	8	
Heptanone	7.791	X	X			X	X	X	X			X		X	X	9	[31]
Amine																	
Indole	11.933	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14	[21,25,31,37,39,41,46,48,49,50,52,59,64]
Quinoline	11.537	X	X	X	X						X					5	[52]
Ester																	
Pentanoate	12.273	X					X		X	X	X		X			6	
Furanone	13.056	X				X		X	X	X	X	X	X	X		9	[52]
Dodecalactone	14.32						X			X	X		X		X	5	
Isopentyl hexanoate	11.176						X		X	X	X		X		X	6	
Alkane																	
Pentadecane	13.145	X	X				X		X	X	X			X		7	[47,55]
Tridecane	11.806	X	X				X		X	X	X					6	[33,47,55]
Tetradecane	9.871	X	X			X	X		X	X	X		X			8	[33,47,55]
Cyclopropane	9.954	X			X		X		X	X			X			6	[58]
Octane	6.033		X				X						X	X	X	5	[8,21,55,56,57,65]
Dithiapentane	7.766							X	X			X	X	X		5	
Alkene																	
Tridecene	11.753	X	X				X		X	X	X					6	
Tetradecadiene	14.18	X	X			X				X	X			X	X	7	
Heptadecene	14.226	X	X		X		X		X	X	X			X	X	9	
Dodecadiene	13.003	X	X				X			X	X					5	
Cyclododecene	14.186	X			X		X	X			X					5	
Carboxylic Acid																	
Benzenepropanoic acid	12.241	X		X			X			X	X		X			6	[47]
Butanoic acid	10.266	X	X	X			X	X	X	X	X	X	X	X	X	12	[25,31,33,36,37,39,42,43,46,48,50,52,55,65]
Pentanoic acid	8.811	X				X		X		X	X		X	X	X	8	[52,53]

(*continued on next page*)

Table 2 (*continued*)

Compound	Retention Time (~)	Week 1		Week 2						Week 3				Week 4		Frequency	Previously Reported Literature
		M1	T1	M1	T1	M2	T2	M3	T3	M2	T2	M3	T3	M3	T3	#	
Amide																	
Piperidinone	11.094	X	X	X						X				X		5	[52]
Thiol																	
Methylbutanethioate	8.511	X	X	X		X	X	X						X		7	
Aromatic																	
Thiophene	10.817	X	X				X			X	X		X		X	7	

Compound	Retention Time (~)	Week 1		Week 2						Week 3				Week 4		Frequency	Previously Reported Literature
		M1	T1	M1	T1	M2	T2	M3	T3	M2	T2	M3	T3	M3	T3	#	
Heptanone	7.791	X	X			X	X	X	X			X		X	X	9	[31]
Amine																	
Indole	11.933	X	X	X	X	X	X	X	X	X	X	X	X	X	X	14	[21,25,31,37,39,41,46,48,49,50,52,59,64]
Quinoline	11.537	X	X	X	X						X	X				5	[52]
Ester																	
Pentanoate	12.273	X					X		X	X	X		X			6	
Furanone	13.056	X				X		X	X	X	X	X	X	X		9	[52]
Dodecalactone	14.32						X			X	X		X		X	5	
Isopentyl hexanoate	11.176						X		X	X	X		X		X	6	
Alkane																	
Pentadecane	13.145	X	X				X		X	X	X			X		7	[47,55]
Tridecane	11.806	X	X				X		X	X	X					6	[33,47,55]
Tetradecane	9.871	X	X			X	X		X	X	X		X			8	[33,47,55]
Cyclopropane	9.954	X			X		X		X	X			X			6	[58]
Octane	6.033		X				X						X	X	X	5	[8,21,55,56,65]
Dithiapentane	7.766							X	X			X	X	X		5	
Alkene																	
Tridecene	11.753	X	X				X		X	X	X					6	
Tetradecadiene	14.18	X	X			X				X	X			X	X	7	
Heptadecene	14.226	X	X		X		X		X	X	X			X	X	9	
Dodecadiene	13.003	X	X				X			X	X					5	
Cyclododecene	14.186	X			X		X	X			X					5	
Carboxylic Acid																	
Benzenepropanoic acid	12.241	X		X			X			X	X		X			6	[47]
Butanoic acid	10.266	X	X	X			X	X	X	X	X	X	X	X	X	12	[25,31,33,36,37,39,42,46,48,50,52,55,65]
Pentanoic acid	8.811	X				X		X		X	X		X	X	X	8	[52,53]
Amide																	
Piperidinone	11.094	X	X	X						X				X		5	[52]
Thiol																	
Methylbutanethioate	8.511	X	X	X		X	X	X						X		7	
Aromatic																	
Thiophene	10.817	X	X				X			X	X		X		X	7	

M = Maggot sample, T = Tissue sample.

Table 1
Detail Summary of pig carcasses used for study.

Pig #	Mass (Kg)	Field Start Date	Pilot/Run
1	0.7	8/17/2018	Pilot
2	0.6	9/4/2018	Pilot
3	1.4	10/1/2018	Run 1
4	1.4	10/1/2018	Run 1
5	1.5	10/16/2018	Run 2
6	1.5	10/16/2018	Run 2
7	0.5	11/4/2018	Run 3
8	0.5	11/4/2018	Run 3

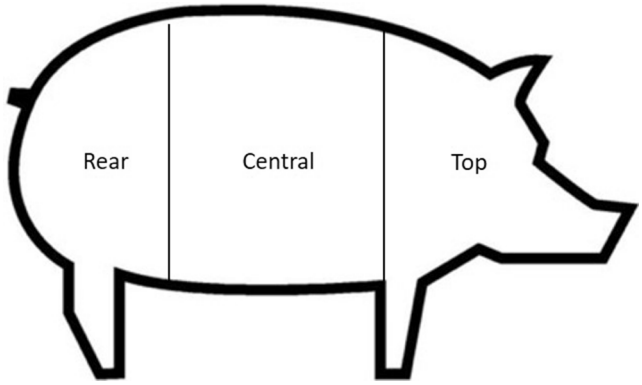

Fig. 1. Schematic of Cadaveric Regions.

cadaveric region as that was where maggots were most abundant during the pilot study. Triplicate maggot samples were collected from each pig along with a tissue sample, for a total of four samples per pig, and a total of eight samples per sampling period. Though the pig carcasses were observed at each of the listed time intervals and environmental conditions were documented, there were times that maggots were not observed therefore not collected. Hence, not every interval yielded maggot samples to collect during this study. Two pigs were used for each experimental test run. Both pigs were laid out at the same time until each was fully decomposed. Samples were collected at the collection times designated from the pilot study.

2.4. Solid phase microextraction (SPME) sampling of volatile organic compounds

To identify the VOCs emitted from collected samples, analysis was completed via SPME-GC–MS. The extraction methodology used to extract the compounds from the maggot matrix was via solid phase microextraction (SPME). The headspace of the maggots was sampled with a 50/30 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber (Supelco, Sigma Aldrich) [31]. Each SPME fiber was conditioned for 30 min at 250 °C at least three times to guarantee each fiber was clean and ready to be used prior to matrix sampling. No external or internal standards were utilized, as the scope of the study was qualitative rather than quantitative analysis.

The collected sample only filled the vial approximately one-fourth of the way full so that the SPME fiber did not interfere with the sample during headspace extraction. Samples were allowed to sit at room temperature for approximately 24 h before the fiber was injected for VOC extraction for equilibrium purposes. The extraction time was approximately 24 h, this equates to the length of time the sample was exposed to the fiber [32].

2.5. Gas chromatography mass Spectrometry analysis (GC–MS)

An Agilent Technologies GC 7890A with an Agilent Technologies 5975C inert XL MSD with triple-axis detector (Agilent Technologies, Santa Clara, CA) was used to separate and analyze the compounds extracted on the SPME fibers. Desorption time of the fiber manually injected in the GC–MS was 8 min at 230 °C [32]. A Rtx®-5 capillary 30 m × 250 μm × 0.25 μm column (Restek Corporation, Bellefonte, PA, USA) was used. Before analyzing the samples, an instrument blank was run on the GC–MS instrument to check its performance with blank vials for calibration purposes. This also validated that the instrument was running correctly prior to sample analyses. The parameters used were similar to those found in a study previously done [32]. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. The oven temperature of 40 °C was held for 5 min then the temperature was increased from 40 °C to 300 °C at a rate of 20 °C/min then held again for 2 min. The total run time for analysis was 20 min. Mass spectra were repeatedly scanned from 45 to 550 amu. Compounds were identified using the NIST 17 (2017) mass spectral reference library. The criteria for the compounds

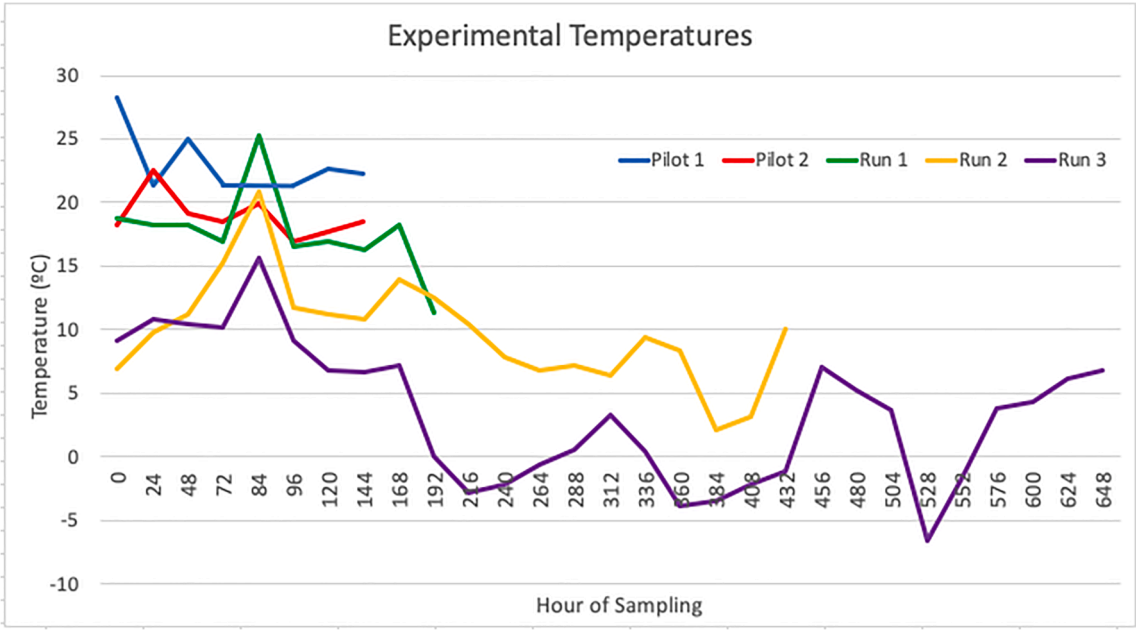

Fig. 2. Temperature Recordings during Carcass Decomposition.

identified were those with detected peaks greater than or equal to a match quality of 80% or above [33].

2.6. Data analysis

All generated data was analyzed using Chemstation software (Agilent Technologies, Santa Clara, CA) and the National Institute of Standards and Technology mass spectral library (NIST 2017) for compound identification. Compounds known to be products of the column or sampling process were not included in the analysis. Statistical analysis was performed using JMP Prop 12.1.0, SAS Institute Inc. 2015. As the study being presented yielded multivariate data from each sample being collected, principal component analysis was utilized. Important variables included not only the type and abundance of compounds being detected for each sample's volatile odor profile, but also the decomposition stage for each collected sample. Peak area responses were unit vector standardized for multivariate analysis. In this case, principal component analysis (PCA) was used to reduce the amount of data using the data's correlation matrix. Using the correlation matrix allowed the data to be standardized having each variable equal to zero mean and unit variance. This meant that the eigenvalues obtained were equal to the number of measured variables. As the variables in this case were the detected compounds, standardization allowed for the variables to be measured with equal weight even though they were obtained throughout different time periods [34]. Compounds retained for data analysis were classified based on their frequency of occurrence. Compounds appearing 10 to 14 times within each sampling occurrence per week were labeled as high frequency occurring compounds. Those compounds occurring 5 to 9 times were labeled as medium frequency occurring compounds, and those occurring less than 4 times were labeled as low frequency occurring compounds. For multivariate statistical analysis, only the high frequency occurring compounds were utilized for calculations. Analysis of variance (ANOVA) was employed to determine whether or not there was significant difference between the amounts of high frequency compounds as a function of decomposition sampling time. All experimental run replicates were combined into the corresponding decomposition sampling timeline to conduct the analysis. The table used for each high frequency compound was broken into two columns – decomposition time and peak area. The duplicate peak areas for each decomposition time were averaged together. This average was then used in the ANOVA. A statistical analysis was performed using JMP Prop 12.1.0, SAS Institute Inc. 2015, running a one-way analysis of variance (ANOVA). Means were used to compare the differences, and Tukey's honestly significant difference (HSD) test was applied to compare the mean values. The significance level for the ANOVA analyses was $p < 0.05$.

3. Results

3.1. Weather conditions

The average temperature during the pilot study was 23.2 °C for pig 1 and 18.9 °C for pig 2. The average temperature during the test runs was 17.7 °C for run 1, 9.8 °C for run 2, and 3.6 °C for run 3. However, these averages do not accurately depict the variation in temperature in Lubbock, Texas. Temperature can increase or decrease by up to 7 °C within a day. Temperature variations across this study can be observed in Fig. 2. During run 1, there was an increase of 8 °C from hour 72 to hour 84 then a decrease of 8 °C by hour 96. During run 2, there was an increase of 5 °C for hour 72 to hour 84 then a decrease of 9 °C by hour 96. During run 3, there was a drop of 7 °C from hour 168 to hour 192. Lower temperatures extended the decomposition process, while warmer temperatures accelerated it. The pilot study measured the warmest temperatures and the carcasses decomposed within six days. Run 3 measured the lowest temperatures, reaching below freezing, resulting in carcass mummification within 27 days.

3.2. Pilot study

The first pilot pig was laid out on August 17, 2018. Maggots were visible at 48 h, making this the first day of collection. Maggot samples as well as tissue samples from the pig were collected until the pig fully decomposed. It took 144 h for pilot pig 1 to fully decompose. Samples were taken from the three regions illustrated in Fig. 2 when available. The pig cadaver fully decomposed and was in the dry/remains stage at hour 144. This process can be seen in Fig. 3.

The average temperature during pilot pig 1 was 23.2 °C, which played a significant role in how quickly the decomposition process occurred. A color chart of the volatile compounds detected from maggots of pilot pig 1 can be seen in the Fig. 4. It is broken down into the three cadaveric regions – top, central, and rear – and highlights the peak area distribution for each volatile compound detected. The most prominent compounds observed in the color chart included: phenol, indole, dimethyl trisulfide, dimethyl disulfide, and phenylethyl alcohol.

Pilot pig 2 was laid out on September 4, 2018, following the removal of pilot pig 1 carcass. It should be noted a different area section of the same land space was used for this placement therefore any residual decomposing matter and remains were absent. It took a total of 120 h for pilot pig 2 to fully decompose. The pig cadaver fully decomposed and was in the dry/remains stage by hour 144. The average temperature during pilot pig 2 was 18.9 °C. A color chart of the volatile compounds detected from maggots of pilot pig 2 was created as a function of sample location site. It is broken down into the three cadaveric regions – top, central, and rear – and highlights the percent of peak area for each volatile compound detected. The most prominent compounds observed in the color chart included: phenol, indole, dimethyl trisulfide, and dimethyl disulfide.

3.3. Test runs-volatile odor collection

A total of two pig carcasses were used for each individual test run of the study. Samples were taken from the central cadaveric region as this was the region with the greatest maggot sample population from the optimization in the pilot runs. A total of 107 compounds were detected in maggot samples and a total of 134 compounds were detected in tissue samples. Of these, a total of 30 compounds were unique to larvae samples which included a range of functional groups to include alcohols, ketones, alkanes, alkenes, acids, aldehydes, and aromatics observed in low frequency. Those compounds included: thietanol, butylated hydroxytoluene, buten-2-one, cycloheptatrien-1-one, pentadic-6-en-2-one, 2-hexadecanone, benzenemethanamine, isoquinoline, pyrazolo [3,4–d]pyrimidin-4-amine, decen-1-yl acetate, cyclopentane, bicyclo [4,1,0]heptane, eicosane, octacosane, heneicosane, naphthalene, trimethylbicyclo[3,1,1]hept-2-ene, cyclohexene, cycloheptene, formic acid, p-toluic acid, chloroacetic acid, octanethioic acid, methylhexanoic acid, eicosadienoic acid, methyl-2-pyridinecarbaldehyde, thieno[2,3–b] thiophene, pyrazine, and arsenous acid. Except for eicosane, which was previously reported in a study by Lunas et al. (20), all other volatiles have never been observed in a maggot sample.

Table 2 highlights only the selected high and medium frequency compounds across the three field runs by evaluating weeks of sampling instead of by hour of sampling. The frequency categorization labeled medium frequency compounds those observed 5 to 9 times throughout the test runs. High frequency compounds included those compounds observed 10 to 14 times throughout the test runs. The high frequency compounds from this table were then the focus of data analysis to analyze any trends observed throughout the study.

Since concurrent tissue samples were collected during the study, a functional group distribution comparison was performed. Fig. 5 depicts the percent of high and medium frequency compounds detected in the two sample matrices across all three test runs. It is highlighted that the functional group distribution across both matrices is very similar. Overall, the blowfly larvae samples had a higher percentage in sulfur,

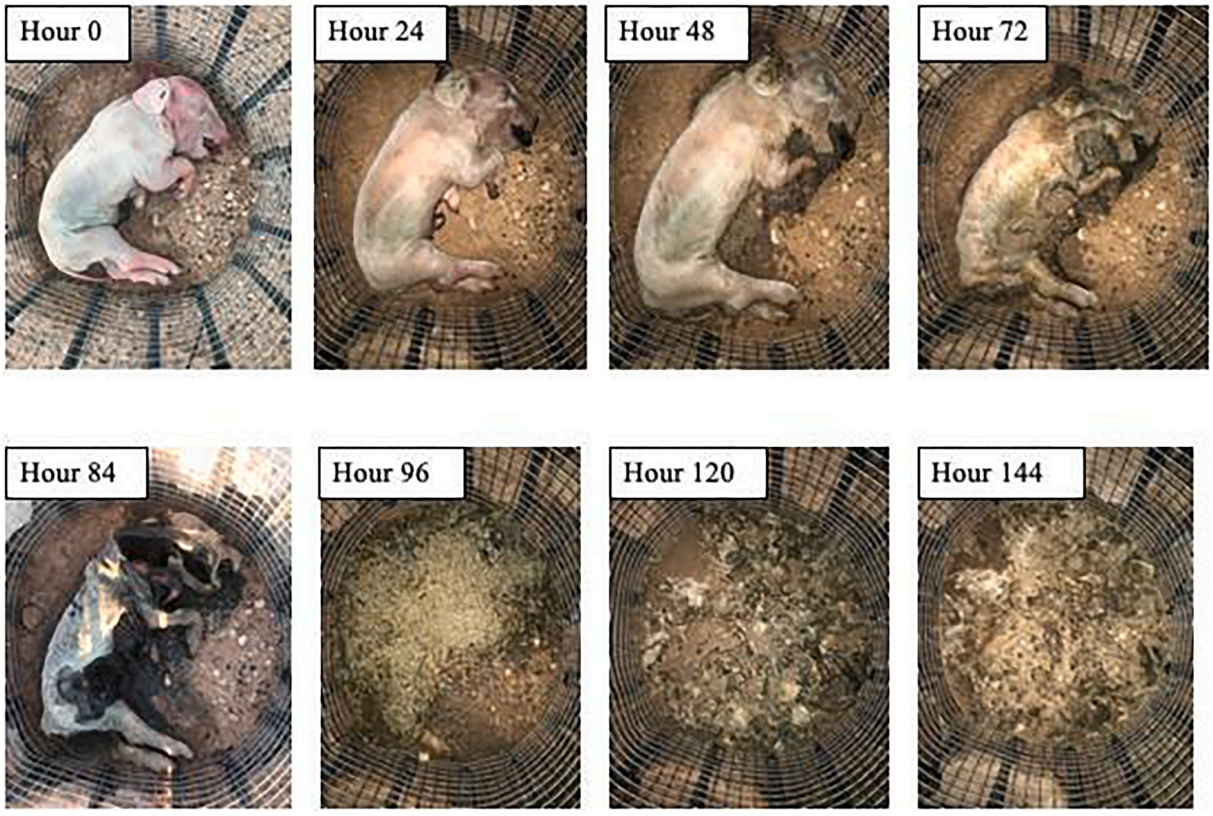

Fig. 3. Decomposition process.

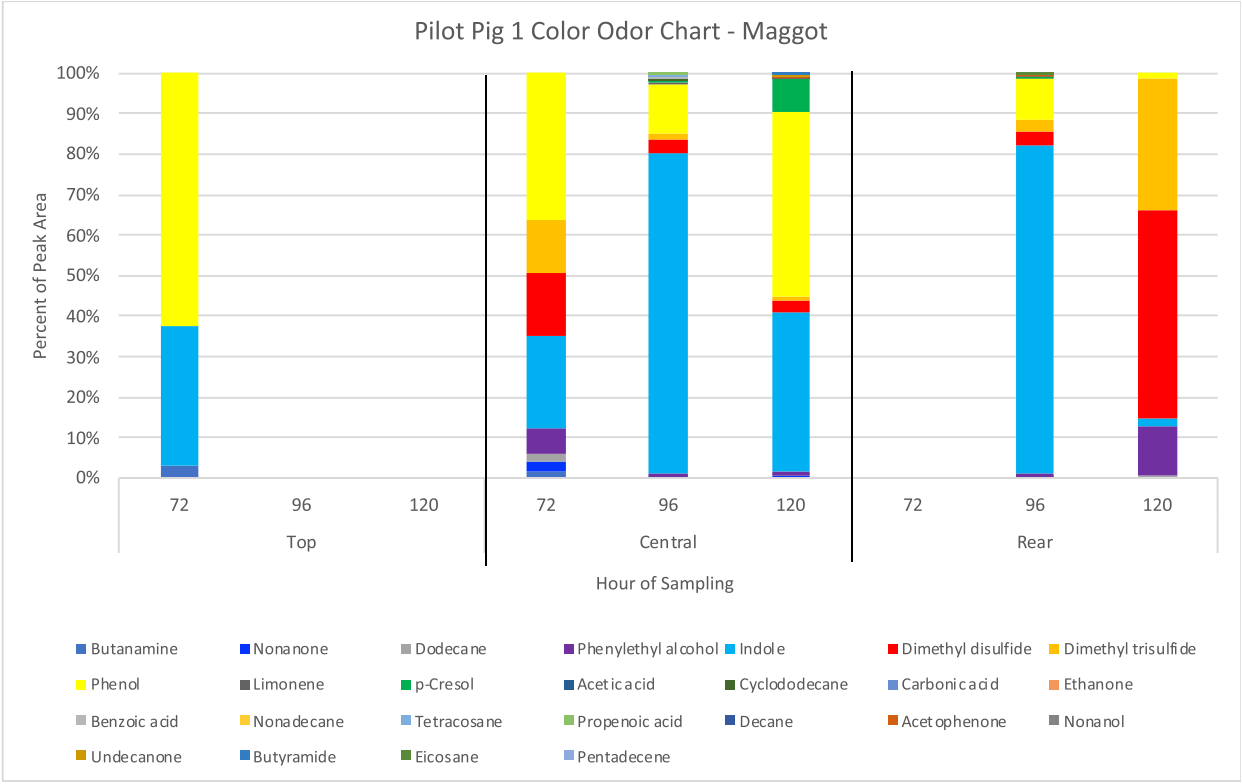

Fig. 4. Pilot Pig Carcass 1– VOC Distribution as a Function of Maggot Location Site.

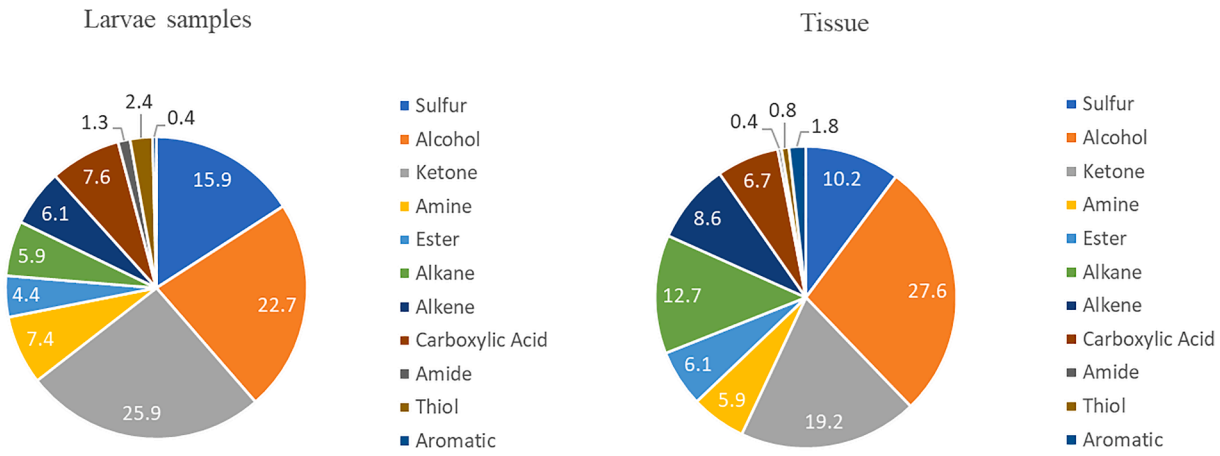

Fig. 5. Comparison of Functional group distribution between blowfly larvae and tissue samples.

ketone, amine, carboxylic acid, and thiol compounds, while the tissue samples had a higher percentage in alcohol, ester, alkane, alkene, and aromatic compounds.

3.4. *High frequency volatile abundance during decomposition process in the larvae sample*

Out of the 10 high frequency compounds, nonanone ($p < 0.001$) and dimethyl disulfide ($p < 0.05$) were the only compounds that had a significant effect with respect to the peak area abundance in relation to sampling time. In the study, sulfur compounds (i.e., dimethyl disulfide and dimethyl trisulfide) along with ketones (i.e., nonanone and undecanone) were at high abundances during the beginning stages of decomposition. As the process continued, alcohols such as phenol were very prominent in the middle stages of decomposition (i.e., bloat and active decay). Indole was also a distinct compound observed, especially during bloating and active decay, as it highlighted an abundance shift between the two stages. In the later stages of decomposition (i.e., advanced decay) phenol and indole were the two compounds observed in highest abundance. The compounds with distinctive patterns observed throughout the decomposition process in this study from the high frequency compounds chosen included: dimethyl disulfide, dimethyl trisulfide, phenol, indole, nonanone, and undecanone. These six compounds displayed abundance changes with respect to decomposition phase throughout each run regardless of the varying environmental conditions as seen in Fig. 6.

The experimental scheme of this study involved collecting multiple larvae odor samples per sampling day. This led to the ability to evaluate trends in the peak area responses of prominent VOCs across multiple runs. Sulfur-containing compounds have previously been reported as one of the most abundant type of chemical class of VOCs in a decomposition odor profile, especially during the early postmortem period [61]. It has been reported that the most likely cause for this type of chemical class among VOCs detected is highly due to breakdown of proteins during decomposition [62] and linked to bacterial action [67]. Oxygenated compounds such as ketones and alcohols are linked to carbohydrate decomposition [62].

3.5. *Principal component analysis*

The VOC profile of the evaluated larvae samples was highly dynamic, with compound abundance and presence shifting across the multiple

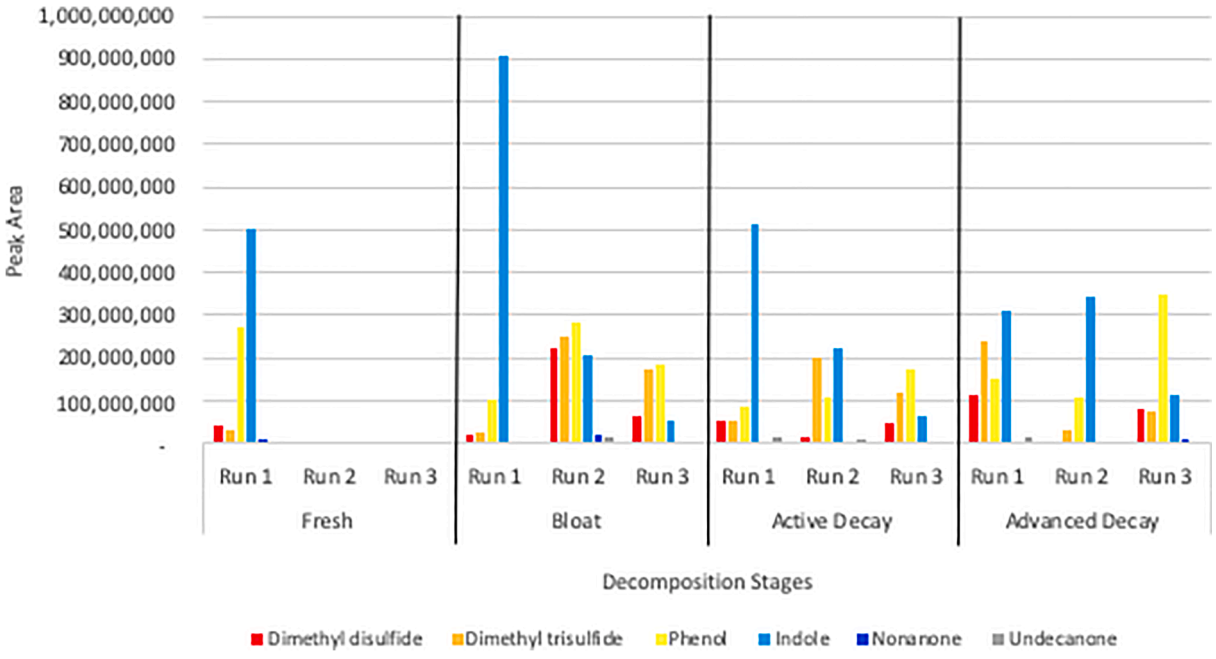

Fig. 6. Odor Profile Patterns of 4 Distinct Compounds Across Decomposition Process.

experimental runs. Thus, the use of multivariate statistical methods was performed to better characterize trends and patterns.

To compare the high frequency odor profiles across time, principal component analysis (PCA) was utilized to monitor the variances in the patterns within the data groups by using a 2-D scatter plot graphing. The PCA plots were used to monitor the larvae odor profile as an evolution of decomposition stage over time using only the 10 high frequency odor volatiles (dimethyl disulfide, dimethyl trisulfide, phenol, phenyl ethyl alcohol, p-cresol, acetophenone, nonanone, undecanone, indole, and butanoic acid). As the runs were performed in distinctive weather conditions, the PCA plots were analyzed for each of the three experimental runs of the study (Fig. 7). As can be observed, the clustering depicted in run 1 classified the volatile odor pattern as separate clusters based on decomposition stage. Principal component 1 had a variation of 29.9% and principal component 2 a variation of 21.5%. Run 2 only had 2 major decomposition stages where larvae were collected, active and advance decay, respectively. The variation of principal component 1 was 32.1% and 25.4% for principal component 2. For run 3, the only clustering that did not have an overlap was the advanced decay, while the bloat and active decay had larvae odor profiles that overlapped taking into consideration the high frequency odor volatile pattern. Principal component 1 had a 29.6% and 20.5% variation for principal component 2. The high frequency selection of chemical odor profile for each larvae sample depicted groups based on the decomposition stage monitored. A trend observed was that as the temperature decreased the volatile odor profile pattern cluster overlaps more across the different decomposition stages.

4. Discussion

The main aim of this study was to evaluate the volatile odor profile from blowfly larvae samples collected from an actively decomposing piglet carcass in outdoor field conditions. The odor profile information from this novel sample specimen may help advance taxonomic tools for postmortem interval determination. This study identified a total of 47 high and medium frequency occurring compounds from larvae samples. Some of these compounds have been previously identified in other decompositional odor profiling studies (see Table 2 for references). This is the first study to evaluate larval masses as an odor source in an active decomposition setting. There is the potential that key odor information

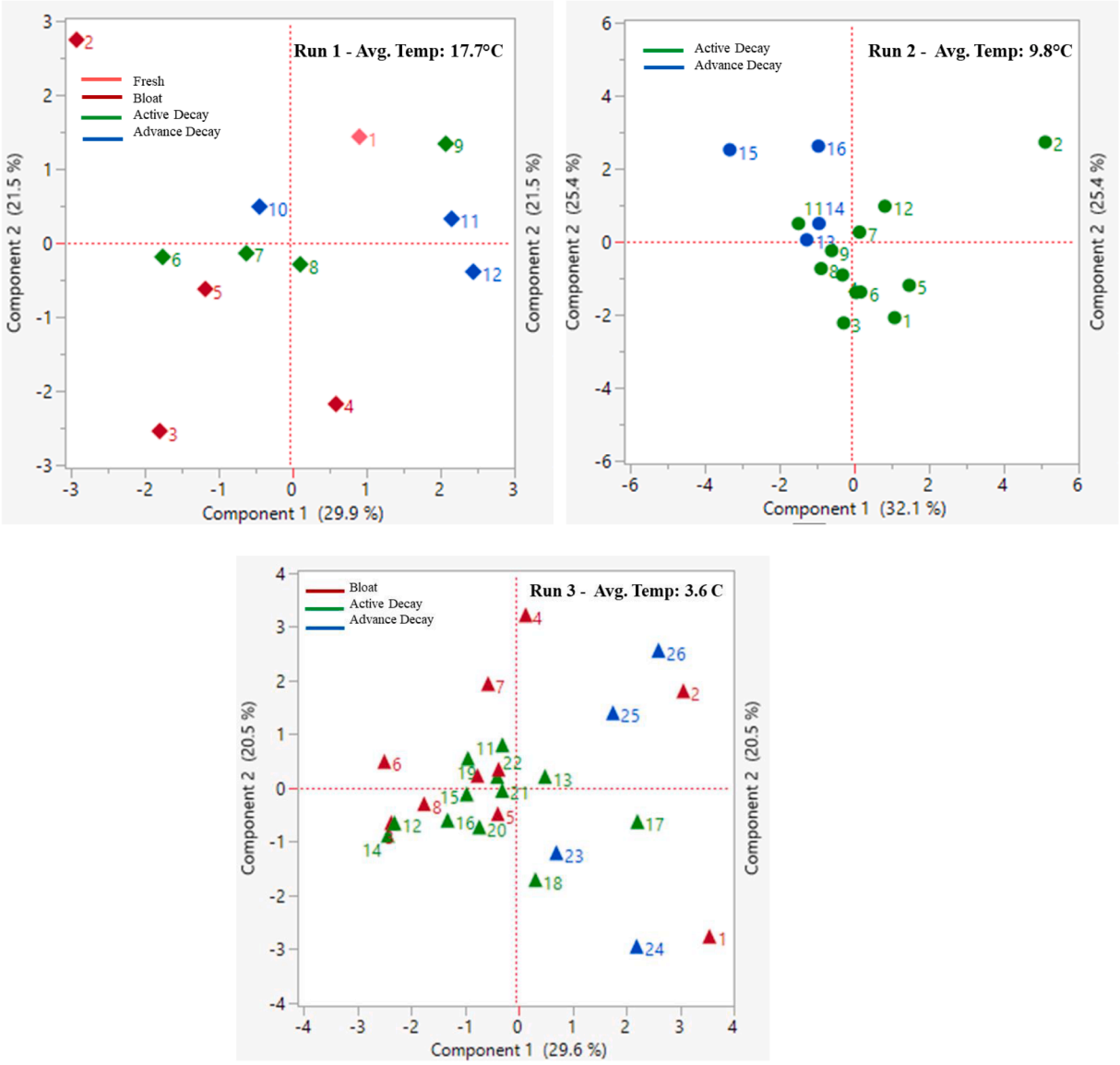

Fig. 7. Principal Component Analysis (PCA) for normalized peak area data from GC–MS; Plots of Calculated Scores for a) Run 1, b) Run 2, and c) Run 3. Numbered labels indicate larvae replicate sample and color code indicates decomposition stage at collection.

from a larvae sample has yet to be fully identified and therefore opens the door to further studies in the future. As it relates to the study of larvae chemical composition, seminal work has targeted perspectives of larvae age and species identification with respect to the emitted VOCs [15,16,17,18,20,21]. While these studies have elucidated the chemical composition of larvae samples as a function of decomposition using laboratory colonies and meat baits, to date there hasn't been a study focusing on blowfly larvae samples collected from an active outdoor cadaveric decomposition model. Characterization of VOCs from our blowfly larvae samples, confirmed results obtained by Frederickx et al. [21] depicting high frequency compounds such as phenol and indole from the maggot mass. Hence, our results offer a preliminary indication that both phenol and indole could very well be indicative of the characteristic larval mass odor picture. This study serves a foundational proof of concept for cases where there is an existence of abundant larvae mass as an additional matrix for analysis. While it is recognized that current decomposition research has focused on whole-body cadaveric volatile odor profiles, our study concurrently evaluated larvae and tissue samples to validate that the decomposition odor volatiles detected were comparable across different sample matrices. The functional group distribution of both matrices was similar thereby corroborating the complex odor picture of cadaveric decomposition. Unique compounds identified in the larval mass were not classified as highly frequent across the sampling, thereby providing an initial indication that corpse putrefactive processes transmit distinctive and abundant odor compounds to the larval mass.

The study characterized a total of ten high frequently occurring VOCs with distinctive patterns observed throughout the decomposition process, corroborating compounds such as dimethyl disulfide, dimethyl trisulfide, phenol, indole, undecanone, nonanone, phenylethyl alcohol, p-cresol, acetophenone, and butanoic acid, which have all been previously reported in the cadaveric odor literature (See Table 2). Not only have these compounds been cited as part of the complex decomposition odor profile but some have also been linked to behavioral responses of necrophagous insects [35]. Several compounds identified in the evaluated larval odor samples including indole, dimethyl disulfide, dimethyl trisulfide, and phenol have been reported as attractant for different species [63,64,68]. Even though this study did not identify unique volatiles emanating from the larvae samples themselves, but rather a parallel odor pattern as those found in decomposing tissue samples, further studies need to investigate the larvae-corpse odor transfer mechanism in order to gain a better understanding of volatile odor origin. In this regard, a recent study has suggested that the blowfly larvae can modify the volatile odor bouquet of a corpse indicating that blowflies prefer to colonize remains which are already colonized by other larval masses [65]. Hence, understanding the odor profile of larvae mass samples may shed light as to what specific VOCs insects use to discriminate corpses and can lead to better knowledge on key semiochemicals important for carrion insects.

Before a tool such as larvae odor profiles be used as a new approach, it is recognized that complex post-mortem environmental microbiomes or introduced post-mortem microbiota must be investigated. It is important to note that the detected VOCs in the larvae samples are inherently impacted by the microbial communities in the studied environment. It has been recognized that carrion microbial communities influence both the physiology and behavioral responses of necrophagous insects through the derivation of volatile molecules [69,70]. In addition, the assembly and performance of these microbial communities during carrion decomposition are affected by a number of factors to include temperature, humidity, tissue type, surrounding vegetation, and soil characteristics [71]. With the dynamic longitudinal trait of microbial communities present on a corpse, there exists a potential for entomological postmortem interval estimation to be altered in terms of larval developmental rates and metrics [72] and consequently on the generated odor collected from such a specimen. As was observed in the PCA plots, during the lower temperature experimental runs there was a decrease in the clustering of the larval odor profile as a function of decomposition stage. This could very well be indicative of the change of microbial community as a result of a drop in temperature, which in turn, affects the amounts of volatiles detected at each stage. Studies have shown that a VOC profile is more complex with warmer decomposition temperatures [66] and more strongly correlated to weather parameters in colder climates [73]. Therefore, the warmer temperature allowed the larval odor samples to fluctuate in compound amounts characteristics of each stage, while the cooler temperatures provided a decreased fluctuation of volatile concentration rendering the clustering to decrease and overlap across stages of decomposition. VOCs emitted by larvae samples are affected not only by endogenous bacterial communities on the remains and larval developmental stage but also by factors such as diet and climate [74,75,76].

In regard to microbial VOC emission by stillborn piglet cadavers, it should be emphasized that newborn vertebrates, including humans, have a different gut microbiome compared to that of adults [77]. Thus, piglet carcasses may decompose differently, and different VOCs may be emitted when compared to a full-sized mature pig [71,78]. It is acknowledged that the carcass samples and analysis conducted in this study is then primarily valid and more directly applicable to young cadavers as a good analog model for newborn or young children. However, the study did provide a sound experimental framework from which to validate the use of larval masses as odor sources, and further studies are needed using animal analogs of comparable human mass. It should be noted that while piglet carcasses were frozen before experimental set-up, the effect of freezing on the study results may be reduced by the fact that neonatal remains already possess reduced gut microbiota, hence not impacting as much as if the carcasses had been of larger mass.

This work has many questions that need to be approached in future studies. Larvae mass samples need to be collected from analog models that resemble the weight of a human corpse. Furthermore, the study needs to be conducted over longer longitudinal timelines to evaluate seasons and yearly climate fluctuations in the odor profile. As with most decomposition studies, the effect of temperature plays a pivotal role in the acquired results, hence a need to monitor multiple years and seasons to expand the collection of larvae samples from multiple species. If an odor profile can be established for a particular specie larval mass, this could ultimately affect the application of volatile odor emissions as indicators of distinctive decomposition stages thereby allowing better estimation on postmortem intervals. It has been recognized that there is an odor transfer between larvae and corpse, however, increasing the number of sampled larval masses can yield a better understanding as to these odor transfer mechanisms. It would be helpful to continuously sample both larvae and decomposing tissue to establish compound identification and abundance on each sample matrix within the decomposition microenvironment.

5. Conclusion

In the present study, larvae samples from the blowfly were collected as potential cadaveric odor sources to analyze the compounds detected at various sampling times throughout the decomposition process. This study was a qualitative (i.e., what compounds were present) and a semi-quantitative (i.e., based on the peak area of each compound) to observe how the volatile odor profiles fluctuated over the sampling time in a dry, arid region in West Texas. Maggot samples were analyzed via headspace SPME extraction and consequently analyzed via GC–MS. Only compounds identified with an 80% in quality abundance from the mass spectral library were recorded for this study.

By comparing larvae samples to decomposing tissue samples, it was concluded that the maggot samples accurately portrayed the decomposition odor profile commonly encountered in cadaveric studies. This provided a foundation that maggots are a viable sample matrix which can be collected off of cadavers for odor biomarker analysis. Furthermore, the high frequency compounds in this study highlighted crucial

variables that affected the acquired odor profile - decomposition time. Compounds such as dimethyl disulfide and nonane had a statistically significant variation of abundance in relation to sampling time. Furthermore, through principal component analysis a preliminary clustering of larvae odor was achieved using only the selected high frequency volatile array that depicted clusters based on decomposition stage. This statistical analysis provided an initial snapshot of how temperature yielded a loss of clustering as the temperature decreased. More research is therefore needed to confirm this trend over longer longitudinal perspectives. However, this study provided an initial perspective on maggot mass VOC characterization from an active decomposition environment under field conditions. Future research will focus on obtaining maggots from human cadavers to further expand and establish the usefulness of this forensic specimen as a PMI estimation tool.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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